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FINAL REPORT

SALMONELLA - ESCHERICHIA COLI/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY WITH A CONFIRMATORY ASSAY WITH DIMETHYLAMINO ETHYLAZIDE (DMAZ)

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14. ABSTRACT

Dimethylamino -2-2ethyl azide (DMAZ, 99.3% pure) was tested for mutagenic potentials with Salmonella typhimurium strains, TA 98, TA 100, TA 1535, TA 1537 and Escherichia coli strain WP2 uvrA by plate incorporation method according to OECD TG 471in compliance with Good Laboratory Practice. The doses tested with Salmonella tester strains and Escherichia coli up to 5000 ?g/plate both in the presence and absence of S9 mix. The results showed that that DMAZ produced positive response in the TA 100 and TA 1537 , both with and without metabolic activation. No positive response was observed with any of the remaining tester strains with and without activation. The confirmatory assay showed similar results with tester strains with and without activation.

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QUALITY ASSURANCE STATEMENT

Salmonella-Escherichia coli/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay with Dimethylamino ethylazide (DMAZ)

The report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc., in accordance with the Good Laboratory Practice regulations as set forth in the Environmental Protection Agency (EPA - TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792 and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17 with any applicable amendments. The following inspections were conducted and the findings reported to the Study Director and study director management. Written status reports of inspections and findings are issued to Covance management according to standard operating procedures.

Inspection Dates			Dates Reported to Study Director and Study Director Management	
From	To	Phase		Auditor
8/25/99	8/25/99	Plating	8/25/99	P. Cáceres
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11/03/99	11/03/99	Final Report Review	11/03/99	K. Groeninger

K. Groeninger — 11/03/99

Representative, Quality Assurance Unit — Date

STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Environmental Protection Agency (EPA - TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792 and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17 with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results, except that the control substances were not fully characterized. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria. All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director.

Study Director:

Michael S. Mecchi

Michael S. Mecchi, MS
Genetic and Cellular Toxicology

11-3-99
Study Completion Date

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ABSTRACT

The objective of this study was to evaluate the test article, Dimethylamino ethylazide (DMAZ), for the ability to induce reverse mutations either in the presence or absence of mammalian microsomal enzymes at 1) the histidine locus in the genome of several strains of *Salmonella typhimurium* and at 2) the tryptophan locus of *Escherichia coli* strain WP2*uvr*A.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding assay using tester strains TA100 and WP2*uvr*A and ten doses of test article ranging from 6.67 to 5,000 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* tester strain WP2*uvr*A. The assay was conducted in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested with the *Salmonella* tester strains were 5,000, 1,000, 333, 100, 33.3, and 10.0 µg per plate in the presence of S9 mix and 5,000, 3,330, 1,000, 333, 100, and 33.3 µg per plate in the absence of S9 mix. The doses tested with tester strain WP2*uvr*A were 5,000, 3,330, 1,000, 333, 100, and 33.3 µg per plate both in the presence and absence of S9 mix. The results of the initial mutagenicity assay were confirmed in an independent experiment.

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, US Army Center for Health Promotion and Preventive Medicine's test article, Dimethylamino ethylazide (DMAZ), did cause positive increases in the mean number of revertants per plate with tester strain TA100 in both the presence (5.2-fold and 6.6-fold) and absence (7.8-fold and 6.6-fold) of microsomal enzymes prepared from Aroclor™-induced rat liver (S9), and with tester strain TA1535 in both the presence (32.6-fold and 24.7-fold) and absence (41.8-fold and 41.3.-fold) of microsomal enzymes prepared from Aroclor™-induced rat liver (S9). No positive increases were observed with any of the remaining tester strain/activation condition combinations.

STUDY INFORMATION

Sponsor
US Army Center for Health Promotion and Preventive Medicine

Test Article
Sponsor's Identification: Dimethylamino ethylazide (DMAZ)
Dimethyl-2-Azidoethylamine, CAS # 86147-04-8
L-15686, Lot 108657P14C5
Date Received: 04/28/99 and 09/14/99
Physical Description: transparent colorless liquid
Storage Conditions: room temperature

Assay Information
Type of Assay: *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay
Protocol No.: 409OECD, Edition 1
Covance Study No.: 20517-0-409OECD

Study Dates
Initiation Date: 08/12/99
Experimental Start Date: 08/18/99
Experimental Termination Date: 09/20/99

Supervisory Personnel
Study Director: Michael S. Mecchi, MS
Laboratory Supervisor: Carlos E. Orantes, BS

OBJECTIVE

The objective of this study was to evaluate the test article and/or its metabolites for their ability to induce reverse mutations either in the presence or absence of mammalian microsomal enzymes at 1) the histidine locus in the genome of several strains of *Salmonella typhimurium* and at 2) the tryptophan locus of *Escherichia coli* strain WP2*uvr*A. The assay design was based on OECD Guideline 471/472, updated and adopted July, 21, 1997.

TEST SYSTEM RATIONALE

The *Salmonella*/Mammalian-microsome reverse mutation assay detects point mutations, both frameshifts and/or base pair substitutions. The strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine

operon. When these histidine-dependent cells (*his*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (*his*+) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *his*+ revertants are readily discernable as colonies against the limited background growth of the *his*- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames Test has been shown to be a sensitive, rapid and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

The *Escherichia coli* WP2*uvr*A reverse mutation assay detects point mutations, specifically base pair substitutions. The *Escherichia coli* tester strain WP2*uvr*A used in this assay is a tryptophan auxotroph (*trp*-) by virtue of a conditionally lethal mutation at a site which blocks a step of tryptophan biosynthesis prior to the formation of anthranilic acid. Since the target site for true back mutation is an ochre nonsense mutation, tryptophan-independent revertants (*trp*+) can arise either by a base change at the site of the original alteration or by suppression by specific suppressor mutations at a second site in tRNA genes (Brusick *et al.*, 1980). When the tryptophan-dependent cells (*trp*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of tryptophan) only those cells which revert to tryptophan independence (*trp*+) are able to form colonies. The trace amount of tryptophan in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *trp*+ revertants are readily discernable as colonies against the limited background growth of the *trp*- cells. While the *trp* reversion system responds to most alkylating agents, base-analog mutagens and certain metals (i.e. soluble chromates), frameshift mutagens would not be expected to be detected by this system.

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames *et al.*, (1975) and Green and Muriel (1976). The assay design is based on the OECD Guideline 471/472, updated and adopted July, 21, 1997.

Test System

Salmonella typhimurium. The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537 as described by Ames *et al.*, (1975). The tester strains in use at Covance were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley. The specific genotypes of these strains are shown in the following table.

Tester Strain Genotypes

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R Factor
TA1535	TA1537		*rfa*	*uvr*B	-
TA100		TA98	*rfa*	*uvr*B	+

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo[a]pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the *uvr*B gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, PKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

Escherichia coli. The tester strain used was the tryptophan auxotroph WP2*uvr*A as described by Green and Muriel (1976). The tester strain in use at Covance was received from The National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

In addition to a mutation in the tryptophan operon, the tester strain contains a *uvr*A DNA repair deficiency which enhances its sensitivity to some mutagenic compounds. This deficiency allows the strain to show enhanced mutability since the *uvr*A repair system would normally act to remove the damaged part of the DNA molecule and accurately repair it afterwards.

Tester strain WP2*uvr*A is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) by base substitution mutagens.

Frozen Permanent Stocks. Frozen permanent stocks of the tester strains were prepared by growing fresh overnight cultures, adding dimethylsulfoxide (DMSO, 0.09 mL/mL of culture) and freezing small aliquots (0.5-1.5 mL) at ≤ −70°C.

Master Plates. Master plates of the tester strains were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with 1) for *Salmonella typhimurium*, an excess of histidine, and biotin, and for tester strains TA98 and TA100, ampicillin (25 µg/mL), to ensure the stable maintenance of the pKM101 plasmid; and 2) for *Escherichia coli*, an excess of tryptophan. Tester strain master plates were stored at 5 ± 3°C.

Inoculation of Overnight Cultures. Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

Harvest of Overnight Cultures. To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensured that cultures had reached a density of at least 0.5 X 10^9 cells per mL and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at 5 ± 3°C.

Confirmation of Tester Strain Genotype. Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay.

***rfa* Wall Mutation.** For the *Salmonella typhimurium* tester strain cultures, the presence of the *rfa* wall mutation was confirmed by demonstration of the sensitivity of the culture to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid. The presence of the pKM101 plasmid was confirmed for the appropriate tester strain cultures by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants. The number of spontaneous revertants per plate in the vehicle controls that is characteristic of the respective strains was demonstrated by plating 100 µL aliquots of the culture along with the appropriate vehicle on selective media.

Culturing Broth. The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Agar Plates. Bottom agar (25 mL per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose.

Overlay Agar for Selection of Revertants. Top (overlay) agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v) and was supplemented with 10 mL of 1) 0.5 mM histidine/biotin solution per 100 mL agar for selection of histidine revertants, or 2) 0.5 mM tryptophan solution per 100 mL of agar for selection of tryptophan revertants. When S9 mix was required, 2.0 mL of the supplemented top agar was used in the overlay. However, when S9 mix was not required, water was added to the supplemented top agar (0.5 mL of water per 2 mL of supplemented top agar) and the resulting 2.5 mL of diluted supplemented top agar was used for the overlay. This dilution ensured that the final top agar and amino acid supplement concentrations remained the same both in the presence and absence of S9 mix.

Test Article

The Sponsor was responsible for the determination of the test article stability and the test article characteristics as defined in the GLP regulations.

Control Articles

Vehicle Controls. Vehicle controls were plated for all tester strains both in the presence and absence of S9 mix. The vehicle control was plated, using a 50 µL aliquot of vehicle (equal to the maximum aliquot of test article dilution plated), along with a 100 µL aliquot of the appropriate tester strain and a 500 µL aliquot of S9 mix (when necessary), on selective agar.

Positive Controls. The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in the following table.

Positive Controls

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	benzo[a]pyrene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	-	sodium azide	2.0 µg
TA1537	+	2-aminoanthracene	2.5 µg
TA1537	-	ICR-191	2.0 µg
WP2*uvr*A	+	2-aminoanthracene	25.0 µg
WP2*uvr*A	-	4-nitroquinoline-N-oxide	1.0 µg

The sources and grades of the positive control articles are as follows:

benzo[a]pyrene (CAS #50-32-8), Sigma Chemical Co., purity ≥98%
2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., purity ≥97%
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., ≥98%
sodium azide (CAS #26628-22-8), Sigma Chemical Co., purity ≥98%
ICR-191 (CAS #1707-45-0), Sigma Chemical Co., purity ≥98%
4-nitroquinoline-N-oxide (CAS #56-57-5), Sigma Chemical Co., purity ≥99%.

Sterility Controls. The most concentrated test article dilution was checked for sterility by plating a 50 µL aliquot (the same volume used in the assay) on selective agar. The S9 mix was checked for sterility by plating 0.5 mL on selective agar.

S9 Metabolic Activation System

S9 Homogenate. Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Batch 0972 (42.8 mg of protein per mL). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per mL in corn oil) at 500 mg/kg as described by Ames *et al.*, (1975).

S9 Mix. The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in the following table.

S9 Mix Components

Component	Amount
H_2O	0.70 mL
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.825M KCl/0.2M $mgCl_2$	0.04 mL
S9 Homogenate	0.10 mL
	1.00 mL

Dose Rangefinding Assay

The growth inhibitory effect (cytotoxicity) of the test article to the test system was determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

Design. The dose rangefinding assay was performed using tester strains TA100 and WP2*uvr*A both in the presence and absence of S9 mix. Ten doses of test article were tested at one plate per dose. The test article was checked for cytotoxicity up to a maximum concentration of 5 mg per plate.

Rationale. The cytotoxicity of the test article observed on tester strain TA100 is generally representative of that observed on the other *Salmonella typhimurium* tester strains and because of the comparatively high number of spontaneous revertants per plate observed with this strain, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain WP2*uvr*A does not possess the *rfa* wall mutation that the *Salmonella typhimurium* strains have and thus, a different range of cytotoxicity may be observed. Also, the cytotoxicity induced by a test article in the presence of S9 mix may vary greatly from that observed in the absence of S9 mix. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the S9 mix.

Evaluation of the Dose Rangefinding Assay. Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

Selection of the Maximum Dose for the Mutagenicity Assay. Since no cytotoxicity was observed in the dose rangefinding assay, the highest dose level of test article used in the mutagenicity assay was the same as that tested in the rangefinding assay.

Mutagenicity Assay

Design. The assay was performed using tester strains TA98, TA100, TA1535, TA1537, and WP2*uvr*A both in the presence and absence of S9 mix along with the appropriate vehicle and positive controls. The doses of test article were selected based on the results of the dose

rangefinding assay. The results of the initial mutagenicity assay were confirmed in an independent experiment.

Frequency and Route of Administration. The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames *et al.*, (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) were combined in molten agar which was overlaid onto a minimal agar plate. Following incubation, revertant colonies were counted. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate.

Plating Procedures
These procedures were used in both the dose rangefinding assay and the mutagenicity assay.

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 µL of tester strain and 50 µL of vehicle or test article dose were added to 2.5 mL of molten selective top agar (maintained at 45 ± 2°C). When S9 mix was required, 500 µL of S9 mix, 100 µL of tester strain and 50 µL of vehicle or test article dose were added to 2.0 mL of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 52 ± 4 hr at 37 ± 2°C. Positive control articles were plated using a 50 µL plating aliquot.

Scoring the Plates
Plates which were not evaluated immediately following the incubation period were held at 5 ± 3°C until such time that colony counting and bacterial background lawn evaluation could take place.

Bacterial Background Lawn Evaluation. The condition of the bacterial background lawn was evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose level. Lawns were scored as 1) normal, 2) slightly reduced, 3) moderately reduced, 4) extremely reduced, 5) absent, or 6) obscured by precipitate. If present on the plates, macroscopic precipitate was scored as slight, moderate or heavy.

Counting Revertant Colonies. The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

DATA

Data Presentation

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables section of this report. The historical control data are presented after the data tables.

Assay Acceptance Criteria

Before assay data were evaluated, the criteria for a valid assay had to be met. The following criteria were used to determine a valid assay:

Tester Strain Integrity.

***rfa* Wall Mutation.** To demonstrate the presence of the *rfa* wall mutation, *Salmonella typhimurium* tester strain cultures exhibited sensitivity to crystal violet.

pKM101 Plasmid. To demonstrate the presence of the PKM101 plasmid, cultures of the appropriate tester strains exhibited resistance to ampicillin.

Characteristic Number of Spontaneous Revertants. To demonstrate the requirement for histidine or tryptophan, the tester strain cultures exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the mean vehicle controls were as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25
WP2*uvr*A	5 - 40

Tester Strain Culture Density. To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures were greater than or equal to 0.5×10^9 bacteria per mL and/or had reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per mL.

Positive Control Values in the Absence of S9 Mix. To demonstrate that the tester strains were capable of identifying a mutagen, the mean value of a positive control for a respective tester

strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity). To demonstrate that the S9 mix was capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 mix for a specific strain was evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

Cytotoxicity. A minimum of three non-toxic doses were required to evaluate assay data.

Assay Evaluation Criteria
Once the criteria for a valid assay had been met, responses observed in the assay were evaluated as follows:

Tester Strains TA98, TA100, and WP2*uvr*A. For a test article to be considered positive, it had to produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535 and TA1537. For a test article to be considered positive, it had to produce at least a 3-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

RESULTS

Test Article Handling
In solubility testing, the test article formed a solution in water at a concentration of 99.7 mg/mL. The test article also formed a solution in DMSO at a concentration of 99.5 mg/mL. Water (Quality Biological, Inc., Lot No. 708008) was selected as the vehicle for this study. At 100 mg per mL, which was the most concentrated stock dilution prepared for the mutagenicity assay, the test article formed a transparent colorless solution. The test article remained a solution in all succeeding dilutions prepared for the mutagenicity assay.

Dose Rangefinding Assay
Doses tested in the mutagenicity assay were selected based on the results of the dose rangefinding assay conducted on the test article using tester strains TA100 and WP2*uvr*A in both the presence and absence of S9 mix with one plate per dose. Ten doses of test article, from 6.67 to 5,000 µg per plate, were tested and the results are presented in Tables 1 and 2. These data were generated in Experiment 20517-A1. No cytotoxicity was observed in either the presence or absence of S9 mix as evidenced by no decrease in the number of revertants per plate and a normal background lawn.

Mutagenicity Assay
The mutagenicity assay results for Dimethylamino ethylazide (DMAZ) are presented in Tables 3 through 8. These data were generated in Experiments 20517-B1 and 20517-C1. The data are presented as individual plate counts (Tables 3, 5, 6, and 8) and as mean revertants per plate ± standard deviation (Tables 4, 5, 7, and 8) for each treatment and control group.

The results of the dose rangefinding study were used to select the doses tested in the mutagenicity assay. The doses tested with the *Salmonella* tester strains were 5,000, 1,000, 333, 100, 33.3, and 10.0 µg per plate in the presence of S9 mix and 5,000, 3,330, 1,000, 333, 100, and 33.3 µg per plate in the absence of S9 mix. The doses tested with tester strain WP2*uvr*A were 5,000, 3,330, 1,000, 333, 100, and 33.3 µg per plate both in the presence and absence of S9 mix.

In the initial mutagenicity assay (Experiment 20517-B1, Tables 3, 4, and 5), all data were acceptable and positive increases in the mean number of revertants per plate were observed with tester strain TA100 in the presence (5.2-fold) and absence (7.8-fold) of S9 mix, and with tester strain TA1535 in the presence (32.6-fold) and absence (41.8-fold) of S9 mix. No positive increases were observed with any of the remaining tester strain/activation condition combinations.

In the confirmatory assay (Experiment 20517-C1, Tables 6, 7, and 8), all data were acceptable and positive increases in the mean number of revertants per plate were observed with tester strain TA100 in the presence (6.6-fold) and absence (6.6-fold) of S9 mix, and with tester strain TA1535 in the presence (24.7-fold) and absence (41.3-fold) of S9 mix. No positive increases were observed with any of the remaining tester strain/activation condition combinations.

All criteria for a valid study were met.

CONCLUSION

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, US Army Center for Health Promotion and Preventive Medicine's test article, Dimethylamino ethylazide (DMAZ), did cause positive increases in the mean number of revertants per plate with tester strain TA100

in both the presence (5.2-fold and 6.6-fold) and absence (7.8-fold and 6.6-fold) of microsomal enzymes prepared from Aroclor™-induced rat liver (S9), and with tester strain TA1535 in both the presence (32.6-fold and 24.7-fold) and absence (41.8-fold and 41.3.-fold) of microsomal enzymes prepared from Aroclor™-induced rat liver (S9). No positive increases were observed with any of the remaining tester strain/activation condition combinations.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time or sent to a storage facility designated by the Sponsor.

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DATA TABLES

TABLE 1 : DOSE RANGEFINDING ASSAY RESULTS

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-A1

DATE PLATED: 18-Aug-99

VEHICLE: Water

DATE COUNTED: 22-Aug-99

	TA100 REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
µg/PLATE	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*
0.00 (Vehicle, 50 µL)	95	1	81	1
Test Article				
6.67	113	1	75	1
10.0	123	1	101	1
33.3	176	1	84	1
66.7	210	1	86	1
100	264	1	96	1
333	365	1	126	1
667	325	1	175	1
1000	338	1	205	1
3330	430	1	418	1
5000	471	1	525	1

* Background Lawn Evaluation Codes:

1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

TABLE 2 : DOSE RANGEFINDING ASSAY RESULTS

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-A1

DATE PLATED: 18-Aug-99

VEHICLE: Water

DATE COUNTED: 22-Aug-99

	WP2*uvrA* REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
µg/PLATE	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*
0.00 (Vehicle, 50 µL)	27	1	25	1
Test Article				
6.67	15	1	23	1
10.0	24	1	15	1
33.3	16	1	31	1
66.7	11	1	21	1
100	20	1	14	1
333	20	1	11	1
667	6	1	12	1
1000	12	1	19	1
3330	27	1	16	1
5000	26	1	26	1

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced
4 = extremely reduced; 5 = absent; 6 = obscured by precipitate
sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

TABLE 3 : MUTAGENICITY ASSAY RESULTS - INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-B1

DATE PLATED: 25-Aug-99 VEHICLE: Water

DATE COUNTED: 30-Aug-99 PLATING ALIQUOT: 50 µL

	DOSE/PLATE	REVERTANTS PER PLATE												BACK-GROUND LAWN*
		TA98			TA100			TA1535			TA1537			
		1	2	3	1	2	3	1	2	3	1	2	3	
MICROSOMES: RAT LIVER														
VEHICLE CONTROL		21	27	22	87	110	112	16	15	18	3	8	7	1
TEST ARTICLE	10.0 µg	23	17	18	151	152	152	70	76	84	9	7	6	1
	33.3 µg	20	24	15	180	202	221	101	128	151	2	4	5	1
	100 µg	20	19	23	224	243	225	148	205	203	11	2	8	1
	333 µg	18	27	23	341	400	362	303	245	325	12	15	6	1
	1000 µg	25	12	20	C	317	431	361	392	267	14	11	10	1
	5000 µg	23	10	21	584	524	501	536	447	580	5	13	6	1
POSITIVE CONTROL**		421	437	402	918	751	862	98	96	111	100	105	112	1
MICROSOMES: NONE														
VEHICLE CONTROL		20	17	25	70	93	88	10	11	15	4	8	9	1
TEST ARTICLE	33.3 µg	15	12	12	75	72	91	16	17	18	3	7	8	1
	100 µg	20	24	9	125	105	104	34	28	32	7	7	3	1
	333 µg	17	16	16	127	141	135	67	84	75	10	2	6	1
	1000 µg	17	19	19	232	285	243	173	176	171	6	15	2	1
	3330 µg	20	11	11	482	373	456	437	377	369	9	7	5	1
	5000 µg	12	12	12	617	687	651	466	500	538	6	13	6	1
POSITIVE CONTROL***		223	158	135	486	552	684	583	500	647	505	382	499	1

** TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

C = No count due to contamination on the plate.

TABLE 4 : MUTAGENICITY ASSAY RESULTS - SUMMARY

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-B1

DATE PLATED: 25-Aug-99 VEHICLE: Water

DATE COUNTED: 30-Aug-99 PLATING ALIQUOT: 50 µL

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION								BACK-GROUND LAWN*
		TA98		TA100		TA1535		TA1537		
		MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	
MICROSOMES: RAT LIVER										
VEHICLE CONTROL		23	3	103	14	16	2	6	3	1
TEST ARTICLE	10.0 µg	19	3	152	1	77	7	7	2	1
	33.3 µg	20	5	201	21	127	25	4	2	1
	100 µg	21	2	231	11	185	32	7	5	1
	333 µg	23	5	368	30	291	41	11	5	1
	1000 µg	19	7	374	81	340	65	12	2	1
	5000 µg	18	7	536	43	521	68	8	4	1
POSITIVE CONTROL**		420	18	844	85	102	8	106	6	1
MICROSOMES: NONE										
VEHICLE CONTROL		21	4	84	12	12	3	7	3	1
TEST ARTICLE	33.3 µg	13	2	79	10	17	1	6	3	1
	100 µg	18	8	111	12	31	3	6	2	1
	333 µg	16	1	134	7	75	9	6	4	1
	1000 µg	18	1	253	28	173	3	8	7	1
	3330 µg	14	5	437	57	394	37	7	2	1
	5000 µg	12	0	652	35	501	36	8	4	1
POSITIVE CONTROL***		172	46	574	101	577	74	462	69	1

** TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

TABLE 5 : MUTAGENICITY ASSAY RESULTS - INDIVIDUAL PLATE COUNTS AND SUMMARY

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-B1

DATE PLATED: 25-Aug-99 VEHICLE: Water

DATE COUNTED: 30-Aug-99 PLATING ALIQUOT: 50 µL

	DOSE/PLATE	REVERTANTS PER PLATE WP2*uvrA*			MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION WP2*uvrA*		BACK-GROUND LAWN*
		1	2	3	MEAN	S.D.	
MICROSOMES: RAT LIVER							
VEHICLE CONTROL		19	14	14	16	3	1
TEST ARTICLE	33.3 µg	18	11	14	14	4	1
	100 µg	20	27	17	21	5	1
	333 µg	21	21	19	20	1	1
	1000 µg	13	19	11	14	4	1
	3330 µg	24	16	14	18	5	1
	5000 µg	28	20	18	22	5	1
POSITIVE CONTROL**		156	164	181	167	13	1
MICROSOMES: NONE							
VEHICLE CONTROL		20	20	23	21	2	1
TEST ARTICLE	33.3 µg	22	8	8	13	8	1
	100 µg	11	23	13	16	6	1
	333 µg	25	21	25	24	2	1
	1000 µg	14	14	16	15	1	1
	3330 µg	11	17	12	13	3	1
	5000 µg	18	17	13	16	3	1
POSITIVE CONTROL***		267	215	239	240	26	1

** WP2*uvrA* 2-aminoanthracene 25.0 µg/plate

*** WP2*uvrA* 4-nitroquinoline-N-oxide 1.0 µg/plate

* Background Lawn Evaluation Codes:

1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

TABLE 6 : MUTAGENICITY ASSAY RESULTS - INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-C1

DATE PLATED: 16-Sep-99 VEHICLE: Water

DATE COUNTED: 20-Sep-99 PLATING ALIQUOT: 50 µL

	DOSE/PLATE	REVERTANTS PER PLATE												BACK-GROUND LAWN*
		TA98			TA100			TA1535			TA1537			
		1	2	3	1	2	3	1	2	3	1	2	3	
MICROSOMES: RAT LIVER														
VEHICLE CONTROL		25	24	15	91	113	83	20	24	17	11	18	13	1
TEST ARTICLE	10.0 µg	24	17	31	133	142	158	76	71	70	10	6	13	1
	33.3 µg	32	26	24	203	222	229	120	152	138	6	7	18	1
	100 µg	17	24	27	268	265	283	176	202	247	12	10	7	1
	333 µg	26	37	49	402	380	359	313	355	330	14	12	11	1
	1000 µg	27	39	20	382	404	401	276	375	305	16	12	12	1
	5000 µg	29	25	39	460	533	896	490	501	491	9	14	11	1
POSITIVE CONTROL**		389	365	357	531	906	672	161	146	131	126	128	123	1
MICROSOMES: NONE														
VEHICLE CONTROL		8	21	10	80	65	72	11	9	6	10	8	10	1
TEST ARTICLE	33.3 µg	9	10	10	61	76	87	13	16	14	7	5	11	1
	100 µg	8	10	13	87	91	90	19	26	23	8	12	1	1
	333 µg	21	20	14	104	118	121	45	28	59	13	6	11	1
	1000 µg	14	15	16	183	177	179	122	114	105	6	12	7	1
	3330 µg	8	11	16	317	346	357	298	284	312	9	12	11	1
	5000 µg	14	14	14	468	485	472	414	364	339	11	10	12	1
POSITIVE CONTROL***		282	310	313	618	587	540	490	504	501	591	562	653	1

** TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

TABLE 7 : MUTAGENICITY ASSAY RESULTS - SUMMARY

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-C1

DATE PLATED: 16-Sep-99 VEHICLE: Water

DATE COUNTED: 20-Sep-99 PLATING ALIQUOT: 50 µL

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION								BACK-GROUND LAWN*
		TA98		TA100		TA1535		TA1537		
		MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	
MICROSOMES: RAT LIVER										
VEHICLE CONTROL		21	6	96	16	20	4	14	4	1
TEST ARTICLE	10.0 µg	24	7	144	13	72	3	10	4	1
	33.3 µg	27	4	218	13	137	16	10	7	1
	100 µg	23	5	272	10	208	36	10	3	1
	333 µg	37	12	380	22	333	21	12	2	1
	1000 µg	29	10	396	12	319	51	13	2	1
	5000 µg	31	7	630	234	494	6	11	3	1
POSITIVE CONTROL**		370	17	703	189	146	15	126	3	1
MICROSOMES: NONE										
VEHICLE CONTROL		13	7	72	8	9	3	9	1	1
TEST ARTICLE	33.3 µg	10	1	75	13	14	2	8	3	1
	100 µg	10	3	89	2	23	4	7	6	1
	333 µg	18	4	114	9	44	16	10	4	1
	1000 µg	15	1	180	3	114	9	8	3	1
	3330 µg	12	4	340	21	298	14	11	2	1
	5000 µg	14	0	475	9	372	38	11	1	1
POSITIVE CONTROL***		302	17	582	39	498	7	602	46	1

** TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

TABLE 8 : MUTAGENICITY ASSAY RESULTS - INDIVIDUAL PLATE COUNTS AND SUMMARY

TEST ARTICLE ID: Dimethylamino ethylazide (DMAZ)

EXPERIMENT ID: 20517-C1

DATE PLATED: 16-Sep-99 VEHICLE: Water

DATE COUNTED: 20-Sep-99 PLATING ALIQUOT: 50 µL

DOSE/PLATE		REVERTANTS PER PLATE WP2*uvrA*			MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION WP2*uvrA*		BACK-GROUND LAWN*
		1	2	3	MEAN	S.D.	
MICROSOMES: RAT LIVER							
VEHICLE CONTROL		19	21	25	22	3	1
TEST ARTICLE	33.3 µg	16	18	13	16	3	1
	100 µg	18	33	22	24	8	1
	333 µg	25	23	21	23	2	1
	1000 µg	22	17	22	20	3	1
	3330 µg	21	21	22	21	1	1
	5000 µg	14	19	12	15	4	1
POSITIVE CONTROL**		349	419	313	360	54	1
MICROSOMES: NONE							
VEHICLE CONTROL		14	15	12	14	2	1
TEST ARTICLE	33.3 µg	27	11	15	18	8	1
	100 µg	19	15	13	16	3	1
	333 µg	18	25	20	21	4	1
	1000 µg	7	18	13	13	6	1
	3330 µg	17	13	26	19	7	1
	5000 µg	19	25	21	22	3	1
POSITIVE CONTROL***		162	247	233	214	46	1

** WP2*uvrA* 2-aminoanthracene 25.0 µg/plate

*** WP2*uvrA* 4-nitroquinoline-N-oxide 1.0 µg/plate

* Background Lawn Evaluation Codes:

1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

HISTORICAL CONTROL DATA FOR BACTERIAL MUTAGENICITY STUDIES

Plate Incorporation Method - Report Period 98J

VEHICLE CONTROLS WITH S9 MIX					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	26.1	102.5	11.8	7.4	15.3
Standard Deviation	6.7	14.4	3.8	2.8	4.3
Maximum	49	147	25	17	30
Minimum	11	61	4	1	5
Count	351	342	273	279	237

VEHICLE CONTROLS WITHOUT S9 MIX					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	15.1	92.8	12.2	6.0	15.1
Standard Deviation	5.0	12.5	5.6	2.7	4.2
Maximum	36	133	48	15	28
Minimum	4	61	3	0	6
Count	318	314	261	266	219

POSITIVE CONTROLS WITH S9 MIX*					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	457.5	838.0	125.7	138.1	388.9
Standard Deviation	60.4	161.7	21.1	30.7	103.7
Maximum	761	1535	215	257	707
Minimum	261	274	74	75	112
Count	227	319	271	278	237

POSITIVE CONTROLS WITHOUT S9 MIX**					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	188.9	609.9	564.6	356.7	306.5
Standard Deviation	39.7	101.9	90.1	98.0	89.2
Maximum	320	980	1053	706	584
Minimum	85	378	365	176	141
Count	300	298	260	266	219

* TA98 benzo(a)pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate
WP2*uvr*A 2-aminoanthracene 25.0 µg/plate

** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate
WP2*uvr*A 4-nitroquinoline-N-oxide 1.0 µg/plate